Description

Alkaline Protease

FIELD OF THE INVENTION

The present invention relates to an alkaline protease and to a gene encoding the same.

BACKGROUND OF THE INVENTION

Protease has long been used in industry, and has found utility in a diversity of fields, including detergents such as laundry detergents, fiber modifying agents, leather processing agents, cosmetic compositions, bath additives, food-modifying agents, and pharmaceuticals. Of these, proteases for detergent use are produced in the largest amounts on an industrial scale. Examples of such known proteases include Alcalase, Savinase (registered trademarks; Novozymes), Maxacal (registered trademark; Genencor), Blap (registered trademark; Henkel), and KAP (Kao Corporation).

The purpose of incorporating protease into a detergent is to degrade protein soil adhering to clothes. Such soil actually is a "complex" soil formed of a plurality of organic and inorganic components, including not only proteins but also lipids originating from sebum, solid particles, and other substances. Therefore, demand has arisen for a detergent having excellent detergency to such complex soil.

Under the above situation, some of the present

inventors had previously discovered several species of alkaline protease which have a molecular weight of about 43,000, exhibit a sufficient casein-degrading activity even in the presence of a fatty acid at a high concentration, and also exhibit excellent detergency not only to proteins but also to complex soils which include sebum and other substances, and filed a patent application therefor (see Patent Publication WO99/18218). Since the discovered alkaline proteases differ from subtilisin (which is a conventionally known serine protease derived from a microorganism belonging to the genus *Bacillus*) in terms of molecular weight, primary structure, enzymological characteristics and resistance to oxidants (the alkaline proteases are strongly resistant to oxidants) their classification into a new subtilisin subfamily has been proposed (see, for example, Saeki *et al.*, Biochem. Biophys. Res. Commun., 279, 313-319, 2000).

In order to industrially mass-produce protease having excellent detergency, productivity thereof must be enhanced. To this end, a variety of methods are envisaged, including selective mutant breeding of enzyme-producing bacteria, and alteration of a gene coding for protease or a gene related to regulation of expression thereof so as to increase the amount of secreted protein. Alternatively, the gene coding for protease is modified so that an enhanced specific activity is obtained. From these viewpoints, the present inventors have previously discovered mutated alkaline protease exhibiting

improved protein secretion ability and enhanced specific activity (Japanese Patent Application Nos. 14-304230, 14-304231, and 14-304232 Application Laid-Open (*kokai*) Nos. 2004-000122 and 2004-057195).

However, further improvement in productivity is desired for producing the enzyme on a large scale at low cost. To answer this, means for improving the amount of secreted protein and specific activity has become of keen interest.

Accordingly, the present invention provides an alkaline protease which has excellent detergency against complex soil, exhibits enhanced protein secretion amount and enhanced specific activity, and can be produced at high productivity.

SUMMARY OF THE INVENTION

The present invention provides an alkaline protease having an amino acid sequence wherein one or more amino acid residues selected from those located at (a) position 15, (b) position 16, (c) position 166, (d) position 167, (e) position 187, (f) position 346, and (g) position 405 of the amino acid sequence of SEQ ID NO: 1, or at positions corresponding to these positions are the following amino acid residues, respectively:

Position (a): histidine,

Position (b): threonine or glutamine,

Position (c): glycine,

Position (d): valine,

Position (e): serine,

Position (f): arginine, and

Position (g): aspartic acid.

The present invention also provides a gene encoding the alkaline protease.

The present invention also provides a vector comprising the gene, and a transformant containing the vector.

The present invention also provides a detergent composition containing the above-described alkaline protease.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows amino acid sequence alignment of protease having 80% or higher homology with the amino acid sequence of SEQ ID NO: 1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an alkaline protease which has for example excellent detergency against complex soil, exhibits enhanced protein secretion amount and enhanced specific activity, and can be produced at high productivity.

The present inventors have searched for a new enzyme which is endowed with the characteristics of the aforementioned alkaline protease and also with improved protein secretion amount and specific activity, and have found that such an enzyme, which is a certain alkaline protease, requires the presence of specified amino acid residue(s) at specified position(s) of the amino acid sequence of the alkaline protease.

The alkaline protease of the present invention has an amino acid sequence wherein one or more amino acid residues selected from those located at (a) position 15, (b) position 16, (c) position 166, (d) position 167, (e) position 187, (f) position 346, and (g) position 405 of the amino acid sequence of SEQ ID NO: 1, or at positions corresponding to these positions are the following amino acid residues, respectively:

(a): histidine, (b): threonine or glutamine, (c): glycine, (d): valine,(e): serine, (f): arginine, and (g): aspartic acid.

Namely, the alkaline protease of the present invention is a protease that has been engineered such that one or more amino acid residues selected from among the above-mentioned positions (a) to (g) of an alkaline protease having an amino acid sequence of SEQ ID NO: 1, or amino acid residue(s) of another alkaline protease at position(s) corresponding to the above-mentioned positions (a) to (g), are specified amino acid residue(s), and may be of a wild type, mutant(s) of the wild type, or mutant(s) created by artificial mutagenesis.

As used herein, "another alkaline protease" may be either a wild type enzyme or a mutant of the wild type enzyme. Preferably, "another alkaline protease" exhibits resistance to oxidants and has a molecular weight of 43,000 ± 2,000 as determined by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis), and as an example thereof, mention may be given of an alkaline protease having such an amino acid

sequence that exhibits 80% or higher homology with the amino acid sequence of SEQ ID NO: 1. More preferably, "another alkaline protease" is an enzyme which has an amino acid sequence that exhibits 80% or higher homology with the amino acid sequence of SEQ ID NO: 1; acts in an alkaline region of pH 8 or higher; exhibits resistance to oxidants; shows 80% or higher residual activity after treatment at 50°C for 10 minutes at pH 10; is inhibited by diisopropylfluorophosphate (DFP) and phenylmethanesulfonyl fluoride (PMSF); and has a molecular weight of 43,000 ± 2,000 as determined by SDS-PAGE. As used herein, the expression "exhibit resistance to oxidants" means that after alkaline protease is left to stand at 30°C for 20 minutes in 20 mM Britton-Robinson buffer (pH 10) containing 50 mM hydrogen peroxide and 5 mM calcium chloride, the alkaline protease maintains a residual activity of at least 50%.

Examples of the "alkaline protease having an amino acid sequence of SEQ ID NO: 1" include KP43 [derived from *Bacillus* sp. KSM-KP43 (FERM BP-6532), Patent Publication WO99/18218]. Examples of the "alkaline protease having an amino acid sequence that exhibits 80% or higher homology with the amino acid sequence of SEQ ID NO: 1" include protease KP9860 (GenBank Accession No. AB046403) [derived from *Bacillus* sp. KSM-kp9860 (FERM BP-6534), Patent Publication WO99/18218]; protease 9865 (GenBank Accession No. AB084155) [derived from *Bacillus* sp. KSM-9865 (FERM P-1592), Japanese Patent Application Laid-Open (*kokai*) No. 2003-199559]; protease E-1

(GenBank Accession No. AB046402) [derived from *Bacillus* No. D-6 (FERM P-1592), Japanese Patent Application Laid-Open (*kokai*) No. 49-71191]; protease Ya (GenBank Accession No. AB046404) [derived from *Bacillus* sp. Y (FERM BP-1029), Japanese Patent Application Laid-Open (*kokai*) No. 61-280268]; protease SD521 (GenBank Accession No. AB046405) [derived from *Bacillus* SD521 (FERM P-11162), Japanese Patent Application Laid-Open (*kokai*) No. 3-191781]; protease A-1 (GenBank Accession No. AB046406) [derived from NCIB12289, Patent Publication WO88/01293]; protease A-2 [derived from NCIB12513, Patent Publication WO98/56927]; mutant proteases described in Japanese Patent Application Laid-Open (*kokai*) Nos. 2002-218989 and 2002-306176; mutants obtained through substitution of position 251 of the amino acid sequence of SEQ ID NO: 1 by asparagine, threonine, isoleucine, valine, leucine or glutamine; mutants obtained through substitution of position 256 of the same amino acid sequence by serine, glutamine, asparagine, valine, or alanine (Japanese Patent Application Laid-Open *(kokai)* 2003-125783); a mutant obtained through substitution of position 65 of the amino acid sequence of SEQ ID NO: 1 by proline; a mutant obtained through substitution of position 101 of the same amino acid sequence by asparagine; mutants obtained through substitution of position 273 of the same amino acid sequence by isoleucine, glycine, or threonine; mutants obtained through substitution of position 320 of the same amino acid sequence by phenylalanine, valine, threonine, leucine, isoleucine, or glycine; mutants

obtained through substitution of position 359 of the same amino acid sequence by serine, leucine, valine, isoleucine, or glutamine, mutants obtained through substitution of position 387 of the same amino acid sequence by alanine, lysine, glutamine, glutamic acid, arginine, or histidine (Japanese Patent Application Application Laid-Open *(kokai)* 2004-000122); mutants obtained through substitution of position 163 of the amino acid sequence of SEQ ID NO: 1 by histidine, aspartic acid, phenylalanine, lysine, asparagine, serine, isoleucine, leucine, glutamine, threonine or valine; mutants obtained through substitution of position 170 of the same amino acid sequence by valine or leucine; mutants obtained through substitution of position 171 of the same amino acid sequence by alanine, glutamic acid, glycine, or threonine (Japanese Patent Application Application Laid-Open *(kokai)* 2004-057195); and an alkaline protease having an amino acid sequence that exhibits a 80% or higher, preferably 87% or more, more preferably 90% or more, still more preferably 95% or more, homology with any of the above listed amino acid sequences.

Homology of amino acid sequences can be preferably determined by the Lipman-Pearson method (Science, 227, 1435, 1985).

"Amino acid residues located at positions corresponding to the positions ......" can be identified by comparing amino acid sequences of alkaline proteases by means of a known algorithm such as the Lipman-Pearson method, to thereby

assign maximum homology to conserved amino acid residues present in the amino acid sequences. When the amino acid sequences of proteases are aligned by means of such method, regardless of insertion or deletion occurred in the amino acid sequences, the positions of the homologous amino acid residues can be determined in each of the proteases. Conceivably, homologous amino acid residues are located at the same positions in the three-dimensional structure of protease, whereby analogous effects are obtained in terms of specific functions of the intended protease.

As shown in FIG. 1, in which amino acid sequences are aligned by means of the aforementioned method, the amino acid residue at "(a) position 15 of the amino acid sequence of SEQ ID NO: 1" is serine. Through use of the method described in the above paragraph, an amino acid residue at a position corresponding to that position can be identified as, for example, asparagine at position 15 in case of protease E-1. In this connection, the amino acid residue at that position is preferably histidine.

The amino acid residue at "(b) position 16 of the amino acid sequence of SEQ ID NO: 1" is serine. Through use of the above-described method, an amino acid residue at a position corresponding to that position can be identified as, for example, asparagine at position 16 in case of protease E-1. Preferably, the amino acid residue at that position is threonine or glutamine.

The amino acid residue at "(c) position 166 of the

amino acid sequence of SEQ ID NO: 1" is asparagine. Through use of the above-described method, an amino acid residue at a position corresponding to that position can be identified as, for example, asparagine at position 165 in case of protease Ya. Preferably, the amino acid residue at that position is glycine.

The amino acid residue at "(d) position 167 of the amino acid sequence of SEQ ID NO: 1" is glycine. Through use of the above-described method, amino acid residue at a position corresponding to that position can be identified as, for example, serine at position 166 in case of protease Ya. Preferably, the amino acid residue at that position is valine.

The amino acid residue at "(e) position 187 of the amino acid sequence of SEQ ID NO: 1" is asparagine. Through use of the above-described method, an amino acid residue at a position corresponding to that position can be identified as, for example, asparagine at position 186 in case of protease SD-521. Preferably, the amino acid residue at that position is serine.

The amino acid residue at "(f) position 346 of the amino acid sequence of SEQ ID NO: 1" is lysine. Through use of the above-described method, an amino acid residue at a position corresponding to that position can be identified as, for example, lysine at position 346 in case of protease KP9860. Preferably, the amino acid residue at that position is arginine.

The amino acid residue at "(g) position 405 of the

amino acid sequence of SEQ ID NO: 1" is asparagine. Through use of the above-described method, an amino acid residue at a position corresponding to that position can be identified as, for example, asparagine at position 405 in case of protease KP9860. Preferably, the amino acid residue at that position is aspartic acid.

Specific examples of the positions and amino acid residues corresponding to (a) position 15, (b) position 16, (c) position 166, (d) position 167, (e) position 187, (f) position 346, and (g) position 405 of the amino acid sequence (SEQ ID NO: 1) of protease KP43 and positions corresponding to these positions are shown below by way of some preferred examples of the aforementioned "another alkaline protease" (Table 1).

Table 1

Position	Protease							
	KP43	KP9860	9865	E-1	Ya	SD-521	A-1	A-2
(a)	Ser15	Ser15	Ser15	Asn15	Asn15	Asn15	Ser15	Asn15
(b)	Ser16	Ser16	Ser16	Asn16	Asn16	Asn16	Ser16	Asn16
(c)	Ser166	Ser166	Ser166	Asn165	Asn165	Asn165	Asn166	Gly165
(d)	Gly167	Gly167	Gly167	Ser166	Ser166	Ser166	Gly167	Ser166
(e)	Asn187	Asn187	Asn187	Asn186	Asn186	Asn186	Asn187	Asn186
(f)	Lys346	Lys346	Lys346	Lys345	Lys345	Lys345	Lys346	Lys345
(g)	Asn405	Asn405	Asn405	Asn404	Asn404	Asn404	Asn405	Asn404

Among the positions (a) to (g) of the amino acid residues of the alkaline protease of the present invention, two or more positions of the positions (a) to (g) may be concurrently selected, so long as the enzyme characteristics remain unchanged. Preferred examples of two or more

positions being selected concurrently are shown below. Amino acids are designated by the three letter codes, and the symbol "+" means an additional substitution.

Specific examples of two substitutions (i.e., substitution taking place at two positions) include Ser15His + Ser16Thr, Ser15His + Ser16Gln, Lys346Arg + Asn405Asp, Asn187Ser + Lys346Arg, and Asn166Gly + Gly167Val, wherein Ser15His + Ser16Gln, Lys346Arg + Asn405Asp, and Asn166Gly + Gly167Val are preferred.

Specific examples of three substitutions (i.e., substitution taking place at three positions) include Ser15His + Ser16Thr + Asn187Ser, Ser15His + Ser16Gln + Asn187Ser, and Asn187Ser + Asn166Gly + Gly167Val, wherein Ser15His + Ser16Gln + Asn187Ser and Asn187Ser + Asn166Gly + Gly167Val are preferred.

So long as the requirements of the present invention are satisfied, four to seven substitutions may take place, and seven substitutions of Ser15His + Ser16Gln + Asn166Gly + Gly167Val + Asn187Ser + Lys346Arg + Asn405Asp is more preferred.

When the alkaline protease of the present invention is a mutant, the alkaline protease before undergoing mutagenesis (which may be referred to as "parent alkaline protease") is either a "protease having an amino acid sequence of SEQ ID NO: 1" or the aforementioned "another alkaline protease." When the parent alkaline protease is subjected to mutation at a predetermined site thereof, the alkaline protease of the

present invention can be obtained. For example, when an amino acid residue at a position selected from the aforementioned positions (a) to (g) of the amino acid sequence of SEQ ID NO: 1 of protease KP43 (or an amino acid residue at a position corresponding to any of the above positions in the amino acid sequence of another alkaline protease) is replaced by another amino acid residue, the alkaline protease of the present invention can be obtained.

The alkaline protease of the present invention may be obtained through, for example, the following steps. Briefly, a cloned gene encoding parent alkaline protease (SEQ ID NO: 2; a gene encoding SEQ ID NO: 1, or a mature enzyme region, is represented by the sequence starting from the 619th codon) is mutated, and by use of the thus-mutated gene an appropriate host bacterium is transformed, followed by culturing of the recombinant host bacterium and collecting the alkaline protease product of the invention from the culture. Cloning of the gene encoding the parent alkaline protease may be carried out through a generally employed gene recombination technique. For example, a method described in Patent Publication WO99/18218 or Patent Publication WO98/56927 may be employed.

Means for carrying out mutagenesis of the gene encoding the parent alkaline protease may be random mutagenesis or site-directed mutagenesis which are commonly performed. More specifically, mutagenesis of the gene may be carried out by use of, for example, a Site-Directed Mutagenesis System

Mutan-Super Express Km kit (Takara). Alternatively, by means of recombinant PCR (polymerase chain reaction; see "PCR Protocols," Academic Press, New York, 1990), an arbitrary sequence of the gene can be replaced by the arbitrary sequence of another gene.

Production of the protease of the present invention by use of the thus-obtained mutant gene may be carried out, for example, by ligating the mutated gene to a DNA vector capable of stably amplifying the gene, to thereby transform host bacteria. Alternatively, the mutant gene may be introduced into chromosomal DNA of a host bacterium capable of stably maintaining the gene. Examples of the host bacterium which satisfies these requirements include bacteria belonging to the genus *Bacillus*, *Escherichia coli*, mold, yeast, and actinomycetes. Any of these microorganisms is inoculated into a culture medium containing an assimilable carbon source, nitrogen source, and other essential nutrients, and culturing is carried out according to a customary method.

From the thus-obtained culture, alkaline protease may be collected and purified by means of customary methods for collecting and purifying enzymes. For example, the culture is subjected to centrifugation or filtration to thereby remove cells, and the enzyme of interest is obtained from the culture supernatant by means of a routine purification technique. The thus-obtained enzyme solution may be employed as is. Alternatively, the enzyme solution may further be subjected to purification, crystallization, powdering, or

granulation, any of which may be carried out according to a known method.

The thus-produced alkaline protease of the present invention for example exhibits oxidant resistance and maintains casein-degrading activity even in the presence of a fatty acid at a high concentration. The alkaline protease has a molecular weight of 43,000 ± 2,000 as determined by SDS-PAGE, and is active within the alkaline region. Moreover, the alkaline protease exhibits newly acquired properties; i.e., improved specific activity and protein secretion amount compared with those of the parent alkaline protease.

Thus, the alkaline protease of the present invention is useful as an enzyme to be incorporated in a variety of detergent compositions.

No particular limitation is imposed on the amount of the protease of the present invention to be incorporated into a detergent composition, so long as the alkaline protease exhibits activity. The preferred amount is 0.1 to 5,000 PU per kg of detergent composition, more preferably 500 PU or less, in consideration of cost and other factors.

The detergent composition of the present invention may further contain a variety of enzymes in addition to the protease of the present invention. Examples of such additional enzymes include hydrolase, oxidase, reductase, transferase, lyase, isomerase, ligase, and synthetase. Of these, preferred enzymes are proteases other than those of

the present invention, cellulase, keratinase, esterase, cutinase, amylase, lipase, pullulanase, pectinase, mannanase, glucosidase, glucanase, cholesteroloxidase, peroxidase, and laccase, among which the proteases, cellulase, amylase, and lipase are more preferred. Examples of the proteases include commercially available ones such as Alcalase, Esperase, Savinase, Everlase, and Kannase (all are resistered trademarks; Novozymes), Properase and Purafect (resistered trademarks; Genencor); and KAP (Kao Corp.) Examples of cellulase include Celluzyme and Carezyme (resistered trademarks; Novozymes); and KAC, alkaline cellulase produced by *Bacillus* sp. KSM-S237 disclosed in Japanese Patent Application Laid-Open (*kokai*) No. 10-313859, and mutated alkaline cellulase disclosed in Japanese Patent Application Laid-Open *(kokai)* 2003-313592 (these are products of Kao Corp.). Examples of amylase include Termamyl and Duramyl (registered trademarks; Novozymes), Purastar (registered trademark; Genencor), and KAM (Kao Corp.). Examples of lipase include Lipolase and Lipolase Ultra (registered trademarks; Novozymes).

When a protease species other than the protease of the present invention is incorporated into a detergent composition together with the protease of the present invention, its amount is preferably 0.1 to 500 PU per kg of detergent composition. When cellulase is incorporated in combination, the amount of cellulase is preferably 300 to 3,000,000 KU per kg of detergent composition, based on the

unit (KU) determined through the enzyme activity determination method described in paragraph [0020] of Japanese Patent Application Laid-Open (*kokai*) No. 10-313859.

When amylase is incorporated in combination, its amount is preferably 50 to 500,000 IU per kg of detergent composition based on the unit (IU) determined through the amylase activity determination method described in paragraph [0040] of Japanese Patent Application Laid-Open (*kokai*) No. 11-43690.

Moreover, when lipase is incorporated in combination, its amount is preferably 10,000 to 1,000,000 LU per kg of detergent composition based on the unit (LU) determined through the lipase activity determination method described in Example 1 of Japanese *Kohyo* (PCT) Patent Publication No. 8-500013.

Known detergent components may be incorporated into the detergent composition of the present invention. Examples of such known detergent components include the following substances.

(1) Surfactant

Generally, a surfactant is incorporated into the detergent composition in an amount of 0.5 to 60 mass%. In particular, the amount of surfactant is preferably 10 to 45 mass% for preparing a powdery detergent composition, and 20 to 50 mass% for preparing a liquid detergent composition. When the detergent composition of the present invention serves as a bleach composition or a detergent composition for

automated dishwasher, a surfactant is typically incorporated in an amount of 1 to 10 mass%, preferably 1 to 5 mass%.

Examples of the surfactant employed in the detergent composition of the present invention include an anionic surfactant, a nonionic surfactant, an amphoteric surfactant, a cationic surfactant, and a combination thereof. Of these, an anionic surfactant and a nonionic surfactant are preferred.

Examples of a preferred anionic surfactant include a sulfate ester salt of C10-C18 alcohol, a sulfate ester salt of an alkoxylated product of C8-C20 alcohol, an alkylbenzenesulfonate salt, a paraffinsulfonate salt, an α-olefinsulfonate salt, an α-sulfo fatty acid salt, and an α-sulfo fatty acid alkyl ester salt or a fatty acid salt. In the present invention, a linear C10-C14 (preferably C12-C14) alkylbenzenesulfonic acid salt is more preferred. The counter ion is preferably an alkali metal or an amine, and sodium and/or potassium, monoethanol amine, or diethanol amine is more preferred.

Examples of a preferred nonionic surfactant include a polyoxyalkylene alkyl (C8-C20) ether, an alkylpolyglycoside, a polyoxyalkylene alkyl (C8-C20) phenyl ether, a polyoxyalkylene sorbitan fatty acid (C8-C22) ester, a polyoxyalkylene glycol fatty acid (C8-C22) ester, and a polyoxyethylene polyoxypropylene block polymer. A more preferred nonionic surfactant is a polyoxyalkylene alkyl ether [having an HLB value (as calculated through the Griffin

method) of 10.5 to 15.0, preferably 11.0 to 14.5] which is obtained by adding 4 to 20 moles of alkyleneoxide (e.g., ethyleneoxide and propyleneoxide) to a C10-C18 alcohol.

(2) Divalent metal ion scavenger

A divalent metal ion scavenger is preferably incorporated into the composition in an amount of 0.01 to 50 mass%, preferably 5 to 40 mass%. Examples of the divalent metal ion scavenger to be employed in the detergent composition of the present invention include a condensed phosphate such as a tripolyphosphate, pyrophosphate, or orthophosphate; an aluminosilicate such as zeolite; a synthesized layered crystalline silicate; a nitrilotriacetate; an ethyenediamineteraacetate; a citrate; an isocitrate; and a polyacetalcarboxylate. Of these, a crystalline aluminosilicate (synthesized zeolite) is more preferred. Among A-type, X-type, and P-type zeolites, A-type zeolite is particularly preferred. The synthesized zeolite preferably has an average primary particle size of 0.1 to 10 μm, more preferably 0.1 to 5 μm.

(3) Alkaline agent

An alkaline agent is preferably incorporated into the composition in an amount of 0.01 to 80 mass%, preferably 1 to 40 mass%. Examples of the alkaline agent which may be incorporated into the detergent in powder form include an alkali metal carbonate such as sodium carbonate (collectively referred to as dense ash or light ash) and an amorphous alkali metal silicate such as JIS No. 1, No. 2, or No. 3.

These inorganic alkaline agents are effective for the formation of the skeleton of particles during drying of the detergent, contributing to production of a detergent of relatively hard particles with excellent flowability. Examples of alkaline agents other than the above-described substances include sodium sesquicarbonate and sodium hydrogencarbonate. A phosphate such as tripolyphosphate also acts as an alkaline agent. Examples of alkaline agents to be employed in a detergent in liquid form include, in addition to the above-described alkaline agents, sodium hydroxide and mono-, di-, or tri-ethanol amine, which can also be employed as a counter ion of a surfactant.

(4) Anti-redeposition agent

An anti-redeposition agent is preferably incorporated into the composition in an amount of 0.001 to 10 mass%, preferably 1 to 5 mass%. Examples of the anti-redeposition agent to be employed in the detergent composition of the present invention include a polyethylene glycol, a carboxylic polymer, a polyvinyl alcohol, and a polyvinyl pyrrolidone. Of these, the carboxylic polymer exerts not only an anti-redeposition effect, but also the effect of scavenging metal ions and the effect of releasing solid soil particles from the clothing into the washing liquid. The carboxylic polymer is a homopolymer or a copolymer of, for example, acrylic acid, methacrylic acid, or itaconic acid. Examples of preferred copolymers include a copolymerized product of any of the above monomers and maleic acid. The copolymer

preferably has a molecular weight of some thousands to 100,000. In addition to the above carboxylic polymers, a polymer such as poly(glycidyl acid salt), a cellulose derivative such as carboxymethyl cellulose, and an aminocarboxylic polymer such as poly(aspartic acid) are also preferred, since these substances function as a metal ion scavenger, a dispersing agent, and an anti-redeposition agent.

(5) Bleaching agent

A bleaching agent such as hydrogen peroxide or a percarbonate is preferably incorporated into the composition, preferably in an amount of 1 to 10 mass%. When such a bleaching agent is employed, teraacetylethylenediamine (TAED) or

a bleaching activator described in, for example, Japanese Patent Application Laid-Open (*kokai*) No. 6-316700 may be incorporated into the composition in an amount of 0.01 to 10 mass%.

(6) Fluorescent agent

Examples of a fluorescent agent which may be incorporated into the detergent composition of the present invention include a biphenyl fluorescent agent (e.g., Tinopal CBS-X) and a stilbene fluorescent agent (e.g., DM-type fluorescent agent). The fluorescent agent is preferably incorporated in an amount of 0.001 to 2%.

(7) Other components

The detergent composition of the present invention may

contain a builder, a softener, a reducing agent (e.g., sulfite), a deformer (e.g., silicone), a perfume, or other additives, which are known in the field of laundry detergents.

The detergent composition of the present invention can be produced through a routine method by using, in combination, the protease product of the present invention obtained through the above-described method and known detergent components as listed above. The form of the detergent may be determined in accordance with its use, and examples of the form include liquid, powder, granules, paste, and solid.

The thus-obtained detergent composition of the present invention can be used as, among others, a laundry detergent, a bleaching agent, a detergent for hard surfaces, a drainpipe detergent, a denture detergent, or a germicidal detergent for medical instruments.

Examples

The following examples further describe and demonstrate embodiments of the present invention. The examples are given solely for the purpose of illustration and are not to be construed as limitations of the present invention.

Example 1

A region of about 2.0 kb up to the stop codon of an alkaline protease structural gene derived from *Bacillus* sp. KSM-KP43 was subjected to error prone PCR by use of a Takara

Taq (product of Takara), which lacks error repair ability , and by use of adequate amounts of manganese sulfate and dimethylsulfoxide, whereby random mutagenesis was introduced. PCR was carried out using primer 1 (SEQ ID NO: 3) and primer 2 (SEQ ID NO: 4) capable of amplifying the above mentioned DNA fragment of about 2.0 kb, wherein primer 1 was a sense primer with *Bam*HI linker at the 5' end, and primer 2 was an antisense primer with linker at the 5' end. In PCR, the template DNA was denatured at 94°C for one minute, followed by 30 cycles of treatment, each cycle consisting of 94°C × one minute, 55°C × one minute, and 72°C × two minutes. The amplified DNA fragments were purified by use of a DNA Product Purification kit (Roche), and the terminal restriction endonuclease linkers were cleaved with *Bam*HI and *Xba*I. The amplified DNA was mixed with plasmid pHA64 which had undergone treatment with *Bam*HI and *Xba*I (see Japanese Patent Application Laid-Open *(kokai)* 2000-287687; *Bam*HI- and *Xba*I-cleaved sites are contained in a downstream region of promoter 64), and subsequently, ligation reaction was carried out with Ligation High (product of Toyobo). Through ethanol precipitation, plasmid was recovered from the ligase reaction mixture. *Bacillus* sp. KSM-9865 (FERM P-18566) serving as the host bacterium was transformed

The KSM-9865 cells which had undergone the transformation step were grown on a skim milk-containing alkaline agar medium [skim milk (Difco) (1% (w/v)), bactotryptone (Difco) (1%), yeast extract (Difco) (0.5%),

sodium chloride (0.5%), agar (1.5%), sodium carbonate (0.05%), and tetracycline (15 ppm)]. Whether or not a mutated protease gene had been introduced to the KSM-9865 cells was determined on the basis of halo formation. The resultant transformants were inoculated into a seed culture medium (5 mL) [6.0% (w/v) polypeptone S, 0.05% of yeast extract, 1.0% of maltose, 0.02% of magnesium sulfate heptahydrate, 0.1% of potassium dihydrogenphosphate, 0.25% of sodium carbonate, and 30 ppm of tetracycline], followed by shaking the culture for 16 hours at 30°C. The seed culture broth (1% (v/v)) was inoculated into a main culture medium (30 mL) [8% polypeptone S, 0.3% of yeast extract, 10% of maltose, 0.04% of magnesium sulfate heptahydrate, 0.2% of potassium dihydrogenphosphate, 1.5% of sodium carbonate anhydrate, and 30 ppm tetracycline], followed by shaking the culture for three days at 30°C.

The resultant culture was subjected to centrifugation, and the protease activity of the culture supernatant was measured. Protease activity was measured by means of a method employing casein as a substrate, and the protein amount was measured by use of a protein assay kit (Wako Pure Chemical Industries, Ltd.). Through comparison between the obtained measurement and a measurement obtained from a culture supernatant of a culture (culturing was performed under the same culture conditions as above) of a transformant harboring a wild type enzyme gene, mutant protease genes showing improved protease activity were selected.

From the selected transformants, plasmid was recovered by use of a High Pure Plasmid Isolation kit (Roche) and subjected to nucleotide sequencing. By use of plasmid DNA (300 ng) as a template, PCR was carried out in a 20 μL reaction system employing a Big Dye DNA sequencing kit (Applied Biosystems). For analysis, a DNA Sequencer (model: 377, Applied Biosystems) was used. As a result, improved protease productivity was found in the following mutants: an enzyme in which serine at position 15 was replaced by histidine, an enzyme in which serine at position 16 was replaced by threonine, a double mutation in which serine at position 15 and serine at position 16 were replaced by histidine and glutamine, respectively, an enzyme in which asparagine at position 166 was replaced by glycine, an enzyme in which glycine at position 167 was replaced by valine, a double mutation in which asparagine at position 166 and glycine at position 167 were replaced by glycine and valine, respectively, an enzyme in which asparagine at position 187 was replaced by serine, an enzyme in which lysine at position 346 was replaced by arginine, an enzyme in which asparagine at position 405 was replaced by aspartic acid, and a double mutation in which lysine at position 346 and asparagine at position 405 were replaced by arginine and aspartic acid, respectively. Of these mutants, those exhibiting particularly improved productivity are a double mutation in which serine at position 15 and serine at position 16 were replaced by histidine and glutamine, respectively, a double

mutation in which asparagine at position 166 and glycine at position 167 were replaced by glycine and valine, respectively, a double mutation in which lysine at position 346 and asparagine at position 405 were replaced by arginine and aspartic acid, respectively, and an enzyme in which asparagine at position 187 was replaced by serine.

A portion of the culture was diluted, and the diluted portion was applied to a DEAE-Toyopearl (Tosoh) equilibrated with 10-mM Tris-HCl buffer containing 2-mM $CaCl_2$ (pH of the buffer system: 7.5). A non-adsorbed fraction was recovered, whereby substantially homogeneous protease was obtained. Protein amount and casein degradation activity were measured for each purified enzyme. The measurements show that improvement in productivity was attained by the mutations introduced, which led to an improved amount of secreted protein (102 to 108%) or an improved specific activity (104 to 121%), on the basis of the secretion amount or the specific activity of a wild type enzyme being taken as 100% (Table 2).

Next, in an attempt to combine the above-described individual mutation sites, recombinant PCR was performed through use of primers 1 to 8 (SEQ ID NOs: 3 to 10) and Pyrobest (Takara), whereby mutants each bearing combinatorial mutation sites were created. Briefly, using as a template a wild type gene or a mutant gene, a DNA fragment having a size of about 700 bp (including the 15th and 16th positions) from the translation initiation site of the alkaline protease

structural gene was amplified with primer 1 (SEQ ID NO: 3) and primer 3 (SEQ ID NO: 5) for combinatorial mutation, to thereby create a mutant. Similarly, a DNA fragment having a size of about 500 bp including positions 166 and 167 was amplified with primer 4 (SEQ ID NO: 6) and primer 5 (SEQ ID NO: 7); a DNA fragment having a size of 400 bp including position 187 was amplified with primer 6 (SEQ ID NO: 8) and primer 7 (SEQ ID NO: 9); and a DNA fragment having a size of about 500 bp (including positions 346 and 405) up to the termination codon of the alkaline protease structural gene was amplified with primer 2 (SEQ ID NO: 4) and primer 8 (SEQ ID NO: 10). Respective mutants were incubated and then assessed for protease productivity. Improvement in productivity was confirmed on S15H/S16Q/K346R/N405D, S15H/S16Q/N187S/K346R/N405D, and S15H/S16Q/N166G/G167V/N187S/K346R/N405D. The results show that improvement in productivity was attained by the enhanced amount of secreted protein (107 to 112%) or an improved specific activity (103 to 115%) in respective mutants, on the basis of the secretion amount or the specific activity of a wild type enzyme being taken as 100% (Table 2).

Table 2

	Relative protein secretion (%)	Relative specific activity (%)
Wild type	100	100
S15H+S16T	102	101
S15H+S16Q	108	104
N166G+G167V	100	121
N187S	101	108
K346R+N405D	102	107
S15H+S16Q+K346R+N405D	112	103
S15H+S16Q+N187S+K346R+N405D	107	113
S15H+S16Q+N166G+G167V+N187S+K346R+N405D	111	115

The above-listed example alkaline protease mutants of the present invention were found to attain either enhanced protein secretion amount with respect to casein or improved specific activity, or both. Except for these new characteristics, they were found to exhibit the characteristics of the parental alkaline protease; i.e., they exhibit oxidant resistance, maintain casein-degrading activity even in the presence of a fatty acid of high concentration, have a molecular weight of 43,000 ± 2,000 as determined by SDS-PAGE, and are active within the alkaline region.

Referential Example

<Protease assay (casein method)>

A 50 mM borate buffer (pH 10.5) (1 mL) containing casein (Hammerstein method: Merck, 1% (w/v)) was maintained at 30°C for five minutes, and subsequently an enzyme solution

(0.1 mL) was added to the buffer, to thereby allow reaction to proceed for 15 minutes. A reaction stopping solution (0.11M trichloroacetic acid / 0.22M sodium acetate / 0.33M acetic acid) (2.0 mL) was added to the resultant reaction mixture, and the mixture was allowed to stand at room temperature for 30 minutes. Thereafter, the filtrate was collected through filtration by use of a Whatman No. 1 filter, and the degradation product was quantified by means of the method described by Lowry, *et al*. Specifically, an alkaline copper solution (1% Rochelle salt: 1% of copper sulfate pentahydrate: 2% of sodium carbonate / 0.1N sodium hydroxide solution = 1:1:100) (2.5 mL) was added to the filtrate (0.5 mL), and the resultant mixture was allowed to stand at 30°C for 10 minutes. Subsequently, to the mixture was added a phenol reagent [obtained by diluting a commercial phenol reagent (Kanto Kagaku) two-fold with deionized water] (0.25 mL), and the resultant mixture was thoroughly stirred and left to stand at 30°C for 30 minutes. Thereafter, the absorbance of the mixture was measured at 660 nm. One unit of protease activity (1 PU) was defined as the amount of enzyme required for producing acid-soluble protein equivalent to 1 mmol of tyrosine per minute under the above reaction conditions.

Example 2

(1) Preparation of detergent

Water (465 kg) was added to a mixing bath (1 m^3) equipped with a stirring paddle. After the temperature of

the water reached 55°C, a 40% (w/v) sodium polyacrylate aqueous solution (135 kg) was added to the water. The resultant mixture was stirred for 15 minutes, and then sodium carbonate (120 kg), sodium sulfate (60 kg), sodium sulfite (9 kg), and a fluorescent dye (3 kg) were added to the mixture. The resultant mixture was further stirred for 15 minutes, and zeolite (300 kg) was added to the mixture, followed by stirring for 30 minutes, to thereby yield a homogenous slurry (the water content of the slurry: 50 mass%). The slurry was sprayed through pressure spray nozzles provided in the vicinity of the top of a spray-drying tower, to thereby yield a granular base (a high-temperature gas was fed at 225°C through a lower part of the spray-drying tower, and discharged at 105°C from the top of the tower).

Subsequently, the thus-obtained granular base (100 parts by mass) was fed to a Lodige mixer (product of Matsuzaka Giken Co., Ltd., capacity: 20 L, equipped with a jacket). While the granular base was stirred by means of rotation of the main shaft (150 rpm), a mixture of a nonionic surfactant (20 parts by mass), sodium linear alkyl (C10-C13) benzenesulfonate (22 parts by mass), a fatty acid (C14-C18) sodium salt (4 parts by mass), polyethylene glycol (2 parts by mass), and water (4 parts by mass) were added to the mixer over three minutes. Thereafter, the resultant mixture was stirred for five minutes. Furthermore, crystalline sodium silicate (20 parts by mass) and zeolite (10 parts by mass) were added to the mixer for surface coating, to thereby yield

a detergent base.

The detergent base (99 mass%) was mixed with example protease granules of the present invention (0.5 mass%) and a perfume (0.5 mass%), to thereby produce an end product, granular detergent A.

(2) Raw materials employed

Nonionic surfactant: Emulgen 108KM (average mole number of ethylene oxide added: 8.5, product of Kao Corporation)

Aqueous solution of sodium polyacrylate: average molecular weight: 10,000 (produced by use of the method described in Examples of Japanese Patent Publication (*kokoku*) No. 2-24283)

Sodium carbonate: Dense ash (product of Central Glass Co., Ltd.)

Zeolite: Zeolite 4A (average particle size: 3.5 μm, product of Tosoh Corporation)

Polyethylene glycol: K-PEG6000 (average molecular weight: 8,500, product of Kao Corporation)

Crystalline sodium silicate: Powder SKS-6 (product of Hoechst Tokuyama)

Example protease granules of the present invention: granules prepared from each of the purified samples of the example alkaline proteases of the present invention shown in Table 2 by use of the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990 (6 PU/g)

Fluorescent dye: Tinopal CBS-X (product of Ciba-Geigy

Corp.)

Example 3

(1) Preparation of detergent

The slurry (solid content: 50 mass%) was spray-dried with 250°C hot air, to thereby yield a granular base containing sodium polyacrylate (mass average molecular weight: 10,000) (7 mass%), sodium carbonate (26 mass%), sodium sulfate (20 mass%), sodium chloride (6 mass%), the fluorescent dye (0.5 mass%), zeolite (40 mass%), and water (0.5 mass%).

Subsequently, the thus-obtained granular base (100 parts by mass) was fed to a Lodige mixer (product of Matsuzaka Giken Co., Ltd., capacity: 20 L, equipped with a jacket). While the granular base was stirred by means of rotation of the main shaft (150 rpm), a mixture of a nonionic surfactant (20 parts by mass), sodium linear alkyl (C10-C13) benzenesulfonate (22 parts by mass), a fatty acid (C14-C18) sodium salt (4 parts by mass), polyethylene glycol (2 parts by mass), and water (4 parts by mass) were added to the mixer over three minutes. Thereafter, the resultant mixture was stirred for five minutes. Furthermore, crystalline sodium silicate (20 parts by mass) and zeolite (10 parts by mass) were added to the mixer for surface coating, to thereby yield a detergent base.

The detergent base (95 mass%) was mixed with bleaching agent granules (2.8 mass%), bleaching activator granules (1.2 mass%), example protease granules of the present invention

(0.5 mass%), and a perfume (0.5 mass%), to thereby produce an end product, granular detergent B.

(2) Raw materials employed

Nonionic surfactant: Emulgen 108KM (average mole number of ethylene oxide added: 8.5, product of Kao Corporation)

Aqueous solution of sodium polyacrylate: average molecular weight: 10,000 (produced by use of the method described in Examples of Japanese Patent Publication (*kokoku*) No. 2-24283)

Sodium carbonate: Dense ash (product of Central Glass Co., Ltd.)

Zeolite: Zeolite 4A (average particle size: 3.5 μm, product of Tosoh Corporation)

Polyethylene glycol: K-PEG6000 (average molecular weight: 8,500, product of Kao Corporation)

Crystalline sodium silicate: SKS-6 (product of Hoechst Tokuyama)

Example protease granules of the present invention: granules prepared from each of the purified samples of the example alkaline proteases of the present invention shown in Table 2 by use of the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990 (6 PU/g)

Fluorescent dye: Tinopal CBS-X (product of Ciba-Geigy Corp.)

Example 4

Liquid detergent compositions (detergents C and D)

shown in Table 3 were prepared.

Table 3

Components	Detergent C (mass%)	Detergent D (mass%)
Nonionic surfactant[1)]	25.0	-
Nonionic surfactant[2)]	5.0	-
Nonionic surfactant[3)]	10.0	-
Nonionic surfactant[4)]	-	9.0
Nonionic surfactant[5)]	-	9.0
Nonionic surfactant[6)]	-	2.5
Anionic surfactant[7)]	1.0	-
Silicone[8)]	-	0.8
Carboxylic acid-based polymer[9)]	2.0	-
Polymer[10)]	-	0.8
Citric acid	0.2	-
Calcium chloride	0.05	-
Monoethanolamine	4.0	-
Triethylene glycol phenyl ether	3.0	-
Propylene glycol	3.0	-
Ethanol	2.0	2.0
Sodium sulfite	0.2	-
Example protease of the present invention[11)]	0.5	1.0
Perfume	0.5	0.5
Water	Balance	Balance
Total	100	100
Concentration upon use	20 g/30 L	40 g/30 L
pH of detergent solution	10.5	7.3

1) Polyoxyethylene (average mole number added: 7) alkyl ether having an alkyl group, derived from a C12-C14 secondary alcohol (Softanol 70, product of Nippon Shokubai Kagaku Kogyo)

2) Polyoxyethylene (average mole number added: 12) alkyl ether having an alkyl group, derived from a C12-C14 secondary alcohol (Softanol 120, product of Nippon Shokubai Kagaku Kogyo)

3) A product obtained by sequentially adding EO

(average mole number: 5), PO (average mole number: 2), and EO (average mole number: 3) to a C10-C14 linear primary alcohol

4) Polyoxyethylene lauryl ether (average mole number of EO added: 8)

5) Polyoxyethylene lauryl ether (average mole number of EO added: 11.5)

6) Narrow range polyoxyethylene alkyl (sec-C_{12}/C_{13}) ether

7) Sodium linear alkyl (C10-C14) benzenesulfonate

8) Amide/ether-modified silicone polymer (BY16-906, product of Dow Corning Toray Silicone Co., Ltd.)

9) A phenoxypolyethylene glycol - acrylic acid - maleic acid copolymer synthesized by use of the method described in lines 6 through 13 of page 11 of Japanese Patent Application Laid-Open (*kokai*) No. 10-60476 (mass average molecular weight: 10,000, solid content: 51.2%)

10) A sodium salt of a pentene/maleic acid (ratio by mol: 50/50) copolymer (mass average molecular weight: 7,000)

11) A purified sample of each of the example alkaline proteases of the present invention shown in Table 2 (15 PU/mL)

Example 5

While sodium percarbonate and sodium carbonate (dense ash) of the components shown in Table 4 below were mixed under stirring, a 40% aqueous solution of sodium polyacrylate and sodium linear alkyl benzenesulfonate, a nonionic surfactant, or sodium lauroyloxybenzenesulfonate were added to the mixture. Subsequently, to the resultant mixture were

added example protease granules of the present invention prepared by use of the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990, and the resultant mixture was stirred until a uniform mixture was attained, whereby a bleaching agent was obtained.

Table 4

Components	Bleaching agent E (mass%)	Bleaching agent F (mass%)
Sodium percarbonate[1)]	72.0	72.0
Sodium carbonate (dense ash)	20.0	20.0
Anionic surfactant[2)]	2.0	-
Nonionic surfactant[3)]	-	2.0
Sodium polyacrylate[4)]	1.0	1.0
Sodium lauroyloxybenzenesulfonate	4.0	4.0
Example protease of the present invention[5)]	1.0	1.0

1) Particle size: 500 to 700 μm

2) Sodium linear alkyl (C12-C14) benzenesulfonate

3) Polyoxyethylene alkyl ether (number of carbon atoms of the alkyl group: 12 to 14, average mole number of EO added: 12)

4) Average molecular weight: 8,000

5) Granules (6 PU/g) prepared from each of the purified samples of the example alkaline proteases of the present invention shown in Table 2 by use of the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990

Example 6

Detergent compositions for an automatic dishwasher (detergents G and H) shown in Table 5 below were prepared.

Table 5

Components	Detergent G (mass%)	Detergent H (mass%)
Pluronic L-61[1]	-	4.0
Softanol EP-7085[2]	4.0	-
Trisodium citrate	-	30.0
Sodium tripolyphosphate	30.0	-
Sodium percarbonate	20.0	20.0
Sodium carbonate	20.0	20.0
Amorphous silicate[3]	10.0	10.0
AA-MA[4]	4.0	4.0
Sodium sulfate	10.0	10.0
α-Amylase[5]	1.0	1.0
Example protease of the present invention[6]	1.0	1.0

1) Polyoxyethylene - polyoxypropylene copolymer (average molecular weight: 2,000)

2) A product obtained by adding to a C12-C14 sec-alcohol ethylene oxide (7 mol) and propylene oxide (8.5 mol)

3) JIS No.2 sodium silicate

4) An acrylic acid - maleic acid copolymer

5) Duramyl 60T (registered trademark; product of Novozymes)

6) Granules (6 PU/g) prepared from each of the purified samples of the example alkaline proteases of the present invention shown in Table 2 by use of the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990

Example 7

A detergent composition for hard surfaces (detergent J) was prepared from components shown in Table 6 below.

Table 6

Components	Detergent J (mass%)
Anionic surfactant[1)]	15.0
Nonionic surfactant[2)]	5.0
Nonionic surfactant[3)]	5.0
Amphoteric surfactant[4)]	7.5
Amphoteric surfactant[5)]	4.0
Citric acid	1.0
Polypropylene glycol[6)]	2.0
Ethanol	5.0
Example protease of the present invention[7)]	1.0
Perfume, water, etc./pH modifier	54.5
Total	100.0

1) Sodium polyoxyethylene (EOP = 4) alkyl (C12) ether sulfate

2) Polyoxyethylene (EOP = 8) alkyl (C12) ether

3) Alkyl (C12) polyglucoside (condensation degree: 1.3)

4) Mono long-chain tertiary alkyl (C12) dimethylamine oxide

5) Alkyl (C12) hydroxydimethyl sulfobetaine

6) Molecular weight: 10,000

7) Each of the purified samples of the example alkaline protease of the present invention shown in Table 2 (15 PU/mL)

Example 8

Granular detergents shown in Table 7 below were prepared by use of the aforementioned detergent A (see Example 2).

Table 7

Components (mass%)	Detergent K	Detergent L	Detergent M	Detergent N
Detergent base of Example 2	98.4	98.3	98.5	97.2
Perfume	0.5	0.5	0.5	0.5
Example protease of the present invention[1)]	0.5	0.5	0.5	0.5
Conventional protease[2)]	0.6			0.6
Cellulase[3)]		0.7		0.7
Lipase[4)]			0.5	0.5

1) Granules (6 PU/g) prepared from each of the purified samples of the example alkaline proteases of the present invention shown in Table 2 by use of the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990

2) Protease K-16 described in Japanese Patent Application Laid-Open (*kokai*) No. 5-25492, the activity thereof having been regulated to 5 PU/g by use of the method described in Example 1 of Japanese Patent Application Laid-Open (*kokai*) No. 62-257990

3) KAC-500 (registered trademark; Kao Corporation)

4) Lipolase 100T (registered trademark; Novozymes)

The present invention enables production of an alkaline protease for example which exhibits activity even in the presence of a fatty acid of high concentration and excellent detergency against complex soils containing not only proteins but also, for example, sebum. Such alkaline protease is secreted efficiently into culture medium, and has high

productivity